Mentored Professional Enrichment Experience

Applicant:

Name of Project/Experience:

Does Increased Serum Undercarboxylated Osteocalcin Induced in Rats by Excess Vitamin D Reflect Impaired Vitamin K Status and Decreased Bone Mineralization?

Location where Project/Experience will take place:

University of Illinios at Urbana-Champaign
Veterinary Medicine Basic Sciences Building
2001 S. Lincoln Ave
Urbana, IL 61802

Mentor Name and Contact Information:

As discussed between Dr. Christopher Masterjohn and Dr. Eric Niederhoffer on 9/30/13, Dr. Masterjohn will serve as the primary mentor while the head of the Comparative Biosciences department, Professor Duncan Ferguson, will serve as the faculty member who oversees the project. Dr. Masterjohn is a Postdoctoral Research Associate, and Professor Ferguson has provided written agreement that he will be promoted to Research Assistant Professor in July, 2014, contingent upon approval of his grant proposal currently under review by the American Heart Association.

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RATIONALE

The dietary recommendations and safety of vitamin D are controversial. Supplementation with vitamin D has increased dramatically over the last decade, yet recent evidence suggests that even moderate excesses of vitamin D are associated with the risk of cardiovascular disease. Vitamin D-induced soft tissue calcification is generally thought to result from hypercalcemia, but vitamin D status is associated with kidney stones and cardiovascular disease in humans even in the absence of hypercalcemia. One hypothesis to explain these findings is that excess vitamin D contributes to soft tissue calcification by leading to the dysregulation of vitamin K-dependent proteins that otherwise protect against this pathological process. A recent study wherein mice were fed calcitriol, a metabolite of vitamin D, supported this hypothesis.

Dr. Masterjohn has provided the first evidence directly supporting this hypothesis in a rat model using dietary vitamins rather than pharmacological metabolites. Rats fed a 50-fold increase in vitamin D over a six-month period developed renal calcification, but rats fed a control diet or three intermediate doses did not. This was accompanied by increased undercarboxylated osteocalcin but not carboxylated osteocalcin in serum (Figure 1). The bone-related mechanisms and consequences of increased serum

undercaroboxylated osteocalcin are unclear. Osteocalcin is a vitamin K-dependent γ-carboxyglutamic acid-containing protein produced by osteoblasts and its carboxylation status is routinely used as a marker of vitamin K adequacy. Although osteocalcin knockout mice were reported to have no detectable alterations in bone mineralization, people with elevated levels of undercarboxylated osteocalcin have a five-fold greater risk of bone fracture, which is corrected by vitamin K supplementation. Thus, the mechanistic role of osteocalcin in bone mineralization is unclear, but elevated serum undercarboxylated osteocalcin may serve as a marker of poor bone mineralization resulting from insufficient vitamin K. Alternatively, since bone resorption leads to the decarboxylation of osteocalcin and its release into serum, an increase in serum undercarboxylated osteocalcin could reflect increased bone resorption. Dr. Masterjohn's results may therefore suggest that vitamin D impairs vitamin K status in bone, but could be confounded by changes in bone resorption, and neither bone mineralization nor the mechanism of increased undercarboxylated osteocalcin has been studied in his model.

The objective of my MPEE project will be to determine what caused the increase in serum undercarboxylated osteocalcin and whether it reflects an impairment in bone mineralization. Dr. Masterjohn snap froze whole hind legs from this study in liquid nitrogen and transferred them to -80°C to preserve them for analysis. I will complete this objective by testing two working hypotheses.

Hypothesis 1: The serum undercarboxylated osteocalcin increased as a result of an increased expression of osteocalcin, increased bone resorption, decreased capacity for vitamin K-dependent carboxylation, or some combination thereof.

Increased expression of osteocalcin in bone could overwhelm the capacity of that tissue for vitamin K-dependent carboxylation and thereby increase undercarboxylated osteocalcin. I will test this by quantifying osteocalcin mRNA expression, protein expression, and carboxylation status in bone.

Bone resorption leads to the decarboxylation of osteocalcin and its release into serum; thus an increase in serum undercarboxylated osteocalcin could reflect increased bone resorption. I will test this hypothesis by measuring the specific bone resorption markers N-telopeptide of type I collagen (NTX) and C-telopeptide type I collagen (CTx) in serum.

It is also possible that the capacity for vitamin K-dependent carboxylation decreased. This seems unlikely because serum carboxylated osteocalcin did not decrease, but I will investigate this possibiltiy if I find decreased carboxylated osteocalcin in bone. In that case, I will test this hypothesis by measuring enzymes necessary for the vitamin-K dependent activation of osteocalcin, including γ-glutamyl carboxylase and vitamin K epoxide reductase (VKOR), as well as vitamin K forms and their metabolites.

Hypothesis 2: The increased serum undercarboxylated osteocalcin reflects poor bone mineralization. This will be investigated by radiographically measuring bone mineral content and by quantifying the elemental calcium content of bone with inductively coupled plasma optical emission spectrometry.

GOALS

My goal is to better understand the mechanisms and interaction between fat-soluble

vitamins A, D and K and to gain practical experience working alongside an expert in the field of nutritional sciences.

Additionally, I expect the data generated from this project to result in a publishable first-author paper. Dr. Masterjohn and I will draft and edit the manuscript together. We expect to carry out this collaboration over email after the project time has been completed.

METHODS

Bone Processing and Osteocalcin: Cortical bone from the midshaft of tibia and femur will be washed of muscle and blood. Samples will be lyophilized, and bones from individual rats will be pulverized with a mortar and pestle. I will use EIA kits to quantify undercarboxylated and carboxylated osteocalcin in bone homogenate following the manufacturer's instructions.

Protein Quantification: For measurements of N-telopeptide of type I collagen (NTX) and C-telopeptide type I collagen (CTX), γ- carboxylase and vitamin epoxide reductase (VKOR), I will use ELISA kits in accordance with the manufacturer's instructions, tested in serum. Phylloquinone, menaquinone-4, and their respective epoxides would be measured by Maret Traber's laboratory at the Linus Pauling Institute at Oregon State University by LC-MS/MS.

Gene Expression: RNA will isolated as described by (1) from long bones. Effects of vitamin D on osteocalcin mRNA expression will be monitored by quantitative PCR.

Calcium Quantification: I will submit samples to the UIUC Veterinary Diagnostic Lab for radiographic determination of bone mineral content and to the Microanalysis Lab for ICP-OES analysis of elemental calcium content.

ANALYSIS

The primary data analysis will use one-way analysis of variance (ANOVA) and pairwise comparisons between group means Bonferonni adjustment for multiple comparisons for all variables. Data will be considered statistically significant with an α of $P<0.05$.

My goals will be met provided the analyses produce reliable and publishable data. If nothing is changed between groups, I will not have resolved the issue, but negative data is still informative. This is unlikely, but in such a case, additional measurements may be completed by Dr. Masterjohn or others under his supervision after the project time has been completed and I will still take primary responsibility together with Dr. Masterjohn for drafting and editing the manuscript.

SUPPORT

1. Do you request support funds? Yes

2. Would you be able to participate if a scholarship is not available? Yes

LITERATURE

1. Gerstenfeld, L. J.C., Franzblau, C. & Sonenshein, G. (1983) *J. Biol. Chem.* 258, 12058.

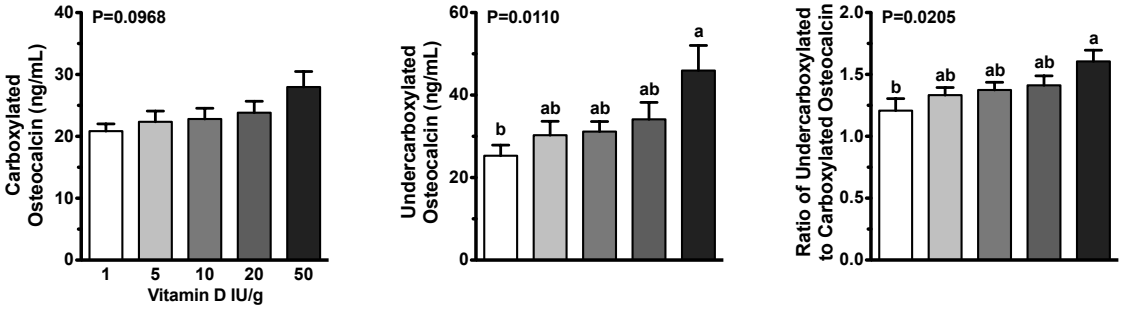

Figure 1. Significant increase in serum undercarboxylated osteocalcin. The P value in the upper left corner shows the results of one-way ANOVA. Different letters above columns indicate a significant ($P<0.05$) difference comparing group means after a Bonferonni correction for all pairwise comparisons (ten comparisons per panel). Diets were fed for six months to initially 5- week-old male Sprague-Dawley rats.